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These studies address the question of how abnormal stromal-epithelial interactions affect the progression of cancer cells. Our studies in mouse mammary gland reveal that ionizing radiation, a known human breast carcinogen, elicits rapid and persistent global changes in the tissue microenvironment. If the microenvironments induced by carcinogens can shape the features and frequency of neoplastic phenotypes, then the carcinogen 'fingerprint' may be envisioned as being built by first laying a foundation of genotypic alterations that expand in the context of a microenvironment that is the result of alterations in stromal and epithelial phenotypes. The current studies are intended to test the hypothesis that *carcinogen-induced changes in the microenvironment constitute a third class of carcinogenic action distinct from those leading to genomic damage or proliferative advantage*. The long-term goal of this research is to determine whether definition of carcinogen-induced microenvironments predicts neoplastic features or frequency. Understanding this aspect of carcinogenesis is important since certain microenvironment alterations might be suitable for therapeutic intervention, which in turn could provide the mean to modify cancer progression.

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## INTRODUCTION

We have proposed the hypothesis that *carcinogen-induced changes in the microenvironment constitute a third class of carcinogenic action distinct from those leading to mutation or proliferative advantage*. Carcinogen-induced microenvironments are postulated to increase the number or susceptibility of epithelial cells to transformation, exert a selective force on initiated cells and/or are conducive to progression. If the microenvironment induced by carcinogens can shape the features and frequency of neoplastic phenotypes, then the carcinogen 'fingerprint' may be envisioned as being built by first laying a foundation of genotypic alterations that expand in the context of a microenvironment that is the result of carcinogen-induced phenotypic change. Understanding this aspect of carcinogenesis is important since certain microenvironment alterations might be amenable to modulation, which in turn could provide the means to modify cancer progression. The proposed studies are intended to obtain further evidence for this hypothesis.

We have studied the effects of a known breast carcinogen, ionizing radiation, on the microenvironment of the mouse mammary gland. We have showed that mouse mammary gland extracellular matrix undergoes rapid and global remodeling that includes the novel expression of tenascin and collagen type III. This remodeling is mediated by the activation of the multipotent cytokine, transforming growth factor- $\beta$ 1 (TGF- $\beta$ ), a potent regulator of both epithelial and stromal function. We have shown that activation can be detected at doses as low as 0.1 Gy and that blocking TGF- $\beta$  with neutralizing antibodies inhibits radiation-induced extracellular matrix remodeling, providing functional confirmation of TGF- $\beta$  activity. Based on these studies, we concluded that exposure to a carcinogen such as radiation can elicit persistent changes in gene expression by non-initiated cells.

By creating chimeric mammary glands consisting of normal or irradiated mammary epithelium in normal or irradiated stroma, we found that the irradiated stroma impedes epithelial maturation. The first aim of the present grant is to test whether radiation-induced TGF- $\beta$  activity regulates this phenotype. The effect of the irradiated stroma may relate to the well-documented age dependence of radiogenic breast cancer. For example, if radiation-induced microenvironment delays the development of the gland in differentiating past a critical check point, then the size or sensitivity of the carcinogen-susceptible population may be increased. Alternatively, radiation-induced TGF- $\beta$  may be a selective force that allows expansion of initiated cells resistant to TGF- $\beta$ . To test whether preneoplastic cells progress more readily in an abnormal stroma, we propose in aim two to create chimeric glands consisting of preneoplastic epithelium in normal versus irradiated stroma.

We predicted that, given the known age dependence of radiogenic mammary cancer in both mice and women, the character of the microenvironment would change as a function of both radiation exposure and mammary development. Our third objective is to compare the radiation-induced microenvironment of adult and immature mice, with particular attention to the expression and activity of TGF- $\beta$ . The regulation of TGF- $\beta$  activation and activity in vivo is not well-understood. We have begun this study by examining the effect of development, hormonal status, and differentiation on TGF- $\beta$  activation.

**Year 2 STUDIES**

**Aim 1: Determine the role of TGF- $\beta$  in the inhibition of mammary gland development by irradiated stroma by using neutralizing antibodies to knockout TGF- $\beta$  activity during outgrowth.**

Due to the new knowledge accruing from the studies in Aims 2 and 3, we have deferred these experiments until year 3 and 4. We specifically wish to identify the pattern of TGF- $\beta$  activation as a function of the estrus cycle in order to optimize administration of the neutralizing antibody.

**Aim 2: Determine the effect of sham versus irradiated fat pads on the carcinogenic potential of epithelia treated with chemical carcinogen, 7,12-dimethylbenz(a)anthracene (DMBA).**

The objective of this aim is to evaluate the role of the irradiated stroma on neoplastic progression, i.e. to determine whether the abnormal stroma "pushes" premalignant cells towards malignancy. In our original proposal, we proposed to combine epithelia treated with an efficient mammary carcinogen, DMBA, with an irradiated stroma in order to test whether the effect of radiation would increase neoplastic progression. Studies in the first year indicated that the COMMA-D mammary epithelial cell line might be a feasible alternative as a preneoplastic epithelial source. COMMA-D cells were derived from a mid-pregnant Balb/c mammary gland. When cultured, they have a mixed morphology and retain the potential to produce differentiated products and when transplanted to cleared syngeneic fat pads are capable of normal outgrowth formation and exhibit low tumorigenicity (Danielson et al., 1984). Although a number of variants have been selected both in vivo and in vitro that are highly tumorigenic (Medina et al., 1987), it has been shown that COMMA-D population is clonal based on characteristic p53 mutations in both alleles (Jerry et al., 1994). We have determined that the neoplastic potential COMMA-D cells could be promoted by transplantation to irradiated stroma (see below).

The testing of the hypothesis that preneoplastic cells progress more readily in an abnormal stroma, is scientifically justified by the fact that COMMA1-D cells harbor defects in both alleles of p53, which is consistent with a preneoplastic population. In addition, the use of COMMA1-D cells is attractive from the view of animal conservation since carcinogen treated-donor animals are not required. And since COMMA-D cells have a known oncogenic genomic change and are well-characterized, it is also a more practical experiment in comparison to carcinogen exposed mammary tissue, whose genotypes will vary from exposure to exposure. **As suggested in the first year progress report review, we provide a modified statement of work using the COMMA1-D cell line as follows.**

**Aim 2: Determine the effect of sham versus irradiated fat pads on the carcinogenic potential of the COMMA1-D mammary epithelial cell line harboring defective p53 genes.**

*Years 1-3*

Task 1: Clear fat pads, house animals until they are 10 weeks of age, irradiate and transplant with COMMA1-D mammary epithelial cells. Evaluate the dependence of tumor formation on cell number injected, COMMA-D culture parameters, period between irradiation and transplantation, radiation dose, and requirement for total versus partial body irradiation.

Task 2: Monitor twice weekly for tumor formation in mammary gland. Record results. Sacrifice as necessary or at 6 weeks for wholemount and histological analysis.

Task 3: Compile data.

Task 4: Repeat experiments.

Task 5: Report results at national meeting and in peer-reviewed journals.

### Preliminary Data: Effect of the Irradiated Stroma on Progression

Progression may be envisioned as induction/selection of cells with genotypic alterations that preferentially expand in the context of a specific microenvironment. Our studies in mouse mammary gland have shown that ionizing radiation elicits rapid and persistent global changes in stromal extracellular matrix composition and growth factor activity. The present studies address the question of whether the irradiated stroma affects the progression of preneoplastic mammary cells. COMMA1-D mammary epithelial cells have a mixed morphology and low tumorigenicity but harbor mutations in both alleles of p53, which leads to constitutive nuclear expression (Figure 1).

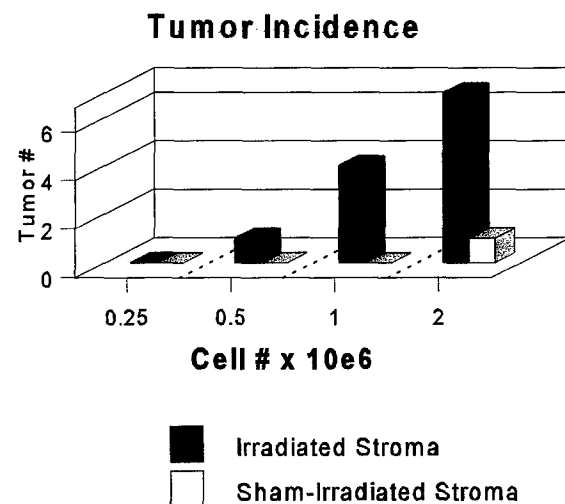
Female Balb/c mammary fat pads were cleared of epithelia at 3 weeks of age. COMMA1-D cells transplanted at this time did not give rise to tumors or to outgrowths. At 10 weeks of age or greater, mice were sham-irradiated or received 4 Gy Co<sup>60</sup>-gamma radiation, whole body. Unirradiated COMMA1-D mammary epithelial cells were transplanted to sham or irradiated cleared fat pads and grown for 6 weeks at which time wholemounts were prepared. Epithelial outgrowths were formed at similar frequencies when transplanted to either control or irradiated tissue. Tumors formed in 1/6 sham-irradiated mammary fat pads transplanted with **Figure 1**  $2 \times 10^6$  COMMA-D cells while 7 tumors were found

in 6 irradiated fat pads. To test whether subpopulations had different tumorigenic potentials, three morphological variants were isolated based on differential trypsinization. All cell populations produced tumors preferentially in the irradiated host suggesting that each population contains, or can give rise to, tumorigenic cells. We postulate that radiation-induced changes in the microenvironment facilitate progression of COMMA1-D cells such that population containing preneoplastic genetic lesions to expands and generates tumors.

### Aim 3: Define the radiation-induced microenvironment as a function of mammary gland developmental status.

We have demonstrated that remodeling of the irradiated mouse mammary gland microenvironment is mediated in part by TGF- $\beta$  (Ehrhart et al., 1997). TGF- $\beta$  is an important regulator of differentiation, proliferation and extracellular matrix composition. It has been postulated to play both a positive and negative role in cancer development and progression, which suggests that determining its physiological regulation and activity in particular tumors may provide interesting targets for therapy [Reiss, 1997].

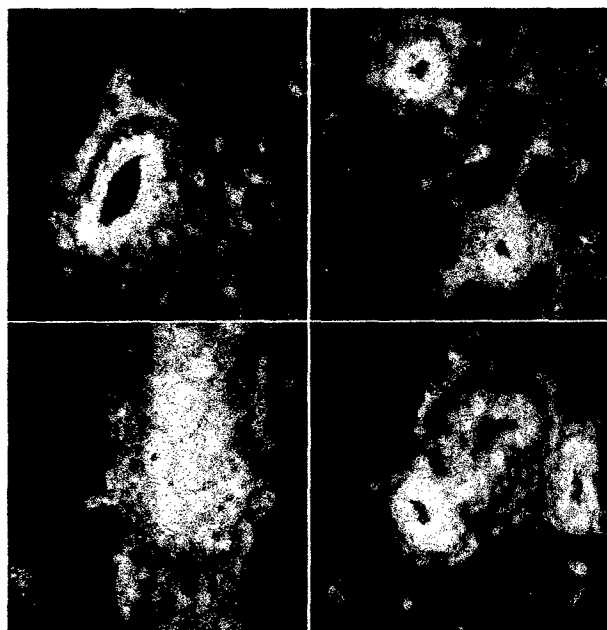
TGF- $\beta$  is secreted as a latent complex that is unable to bind to TGF- $\beta$  receptors until the biologically



active 24-kD mature TGF- $\beta$  is released; this activation is considered to be the critical regulatory event for TGF- $\beta$  function. Radiation exposure elicits rapid and persistent activation of TGF- $\beta$  *in vivo*. We postulated that aberrant TGF- $\beta$  activation by ionizing radiation affects mammary gland development and neoplastic progression by perturbing the balance between the stroma and epithelium.

The effects of TGF- $\beta$  in the mammary gland are complex and not well understood. In order to better understand the role of TGF- $\beta$  during mammary gland growth and development prior to examining the effect of developmental status on the response to radiation, we examined the distribution and abundance of active TGF- $\beta$  in Balb/c mice during normal mammary development. Using an immunostaining protocol that preserves endogenous latent TGF- $\beta$  and antibodies that discriminate between latent and active TGF- $\beta$ , we determined that in normal adult mammary gland latent TGF- $\beta$  is abundant but active TGF- $\beta$  is restricted to epithelial structures.

**Discordant LAP and active TGF- $\beta$  immunoreactivity in the adult mammary luminal epithelium suggests that TGF- $\beta$  activation is regulated.** Antibodies to LAP (green) to localize



**Figure 2**

LTGF- $\beta$ , antigen-purified TGF- $\beta$  antibodies that specifically detect active TGF- $\beta$  (red) and DAPI stained nuclei (blue) were visualized using tricolor digital fluorescence microscopy (Figure 2). Colocalization of LAP and TGF- $\beta$  appears yellow. Discordant localization of antibodies directed to different regions of the same gene product is consistent with the hypothesis that a TGF- $\beta$  antigen is masked in the latent complex but revealed upon activation (Barcellos-Hoff et al., 1994; Ehrhart et al., 1996). Although LAP immunoreactivity (green) was relatively uniform, a checkerboard pattern of TGF- $\beta$  staining (yellow-orange) was observed in the epithelium of certain adult mammary glands. We examined mammary glands from a variety of physiological states to determine the physiological determinants of this pattern.

**Active TGF- $\beta$  immunostaining during postnatal development is associated with proliferation.**

We have correlated the frequency of TGF- $\beta$  positive cells with physiological stages of mammary gland development. During ductal morphogenesis in puberty, endbuds are morphological structures that invade the adipose stroma to establish the ductal tree. All cells of the stroma and epithelium stained with the antibody to LAP. Frequent cells within endbuds show strong TGF- $\beta$  immunostaining. Cap cells at the interface between the endbud and adipose stroma were negative for TGF- $\beta$ . Adjacent sections stained with antibodies to PCNA demonstrated that proliferating cells were common in both the cap and body cells. Ductal epithelium proximal to the region of endbuds show few TGF- $\beta$  positive cells and minimal proliferation (not shown). The association of TGF- $\beta$  with a proliferating structure is paradoxical in view of its postulated role in growth inhibition. The gradient of frequent TGF- $\beta$  positive cells in endbud epithelium to few cells in the ductal epithelium in the flanking region is also paradoxical in terms of growth. Bresciani noted that endbud epithelium is stimulated to proliferate by both estrogen and progesterone but the ductal epithelium derived from



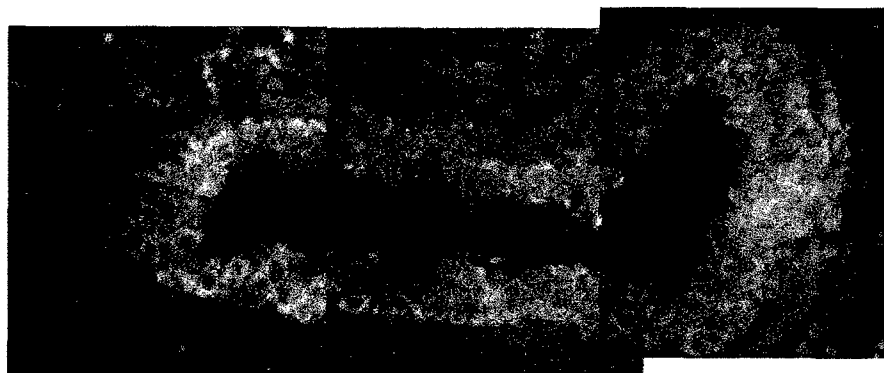


Figure 3

responds only to progesterone (Bresciani, 1968). Similarly, progesterone induced lobular-alveolar differentiation is ductal even when endbuds are present (Silberstein et al., 1996). It has been suggested that there is a modifying factor produced in the immediate vicinity of hormone responsive cells of the endbud.

**Active TGF- $\beta$  immunostaining during the estrus cycle is highly heterogeneous.** The intensity and distribution of TGF- $\beta$  in mammary glands of adult animals show dramatic variation as a function of the estrus cycle (Figure 4). In diestrus, most of the epithelium is faintly, yet evenly stained. During proestrus, a transition occurs in which epithelial structures show highly heterogeneous TGF- $\beta$  staining, some with brilliantly stained cells (top panel), while others show diminished staining (bottom panel). During estrus, the heterogeneity of TGF- $\beta$  immunostaining increases, such that most epithelial structures showed intensely positive cells adjacent to negative cells (top panel represents two cross sections of intact ducts, in which many cells are completely negative). A few structures appear to have either begun to revert or persisted with the low homogeneous staining seen in diestrus (bottom panel).

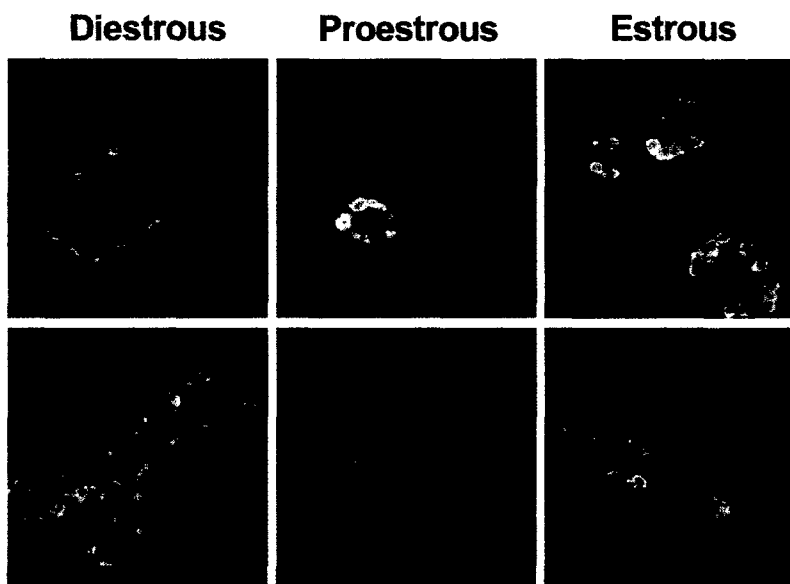


Figure 4

Thus, during mammary development the distribution and intensity of active TGF- $\beta$  and LAP immunoreactivity are highly modulated. Surprisingly, the most intense active TGF- $\beta$  staining is observed during periods of morphogenesis and proliferation while quiescent tissue, like diestrus, exhibit less intense staining. Further, intense TGF- $\beta$  staining is accompanied by down regulation in adjacent cells. We postulate that the heterogeneous intense staining represents a distinct epithelial subpopulation whose nature remain to be determined. One possibility is that these cells have different hormonal sensitivities that might be reflected by estrogen or progesterone receptor expression.

Another explanation is that they represent populations that cooperate during lobular-alveolar morphogenesis. The basis for their distribution can only be determined by systematic analysis of population features.

**Estrogen and progesterone show distinct effects on TGF- $\beta$  mammary epithelial immunoreactivity in ovariectomized mice.** We asked whether the distribution of TGF- $\beta$  positive cells was affected by the ovarian hormones, estrogen and progesterone, following ovariectomy for 14 days (Figure 5). Both LAP and TGF- $\beta$  in mammary epithelium of ovariectomized animals (A, D) were decreased by estrogen treatment for 3 days (B). Estrogen treatment of intact mice also showed greatly reduced staining (C). In contrast, progesterone treatment for 3 days (E) resulted in elevated

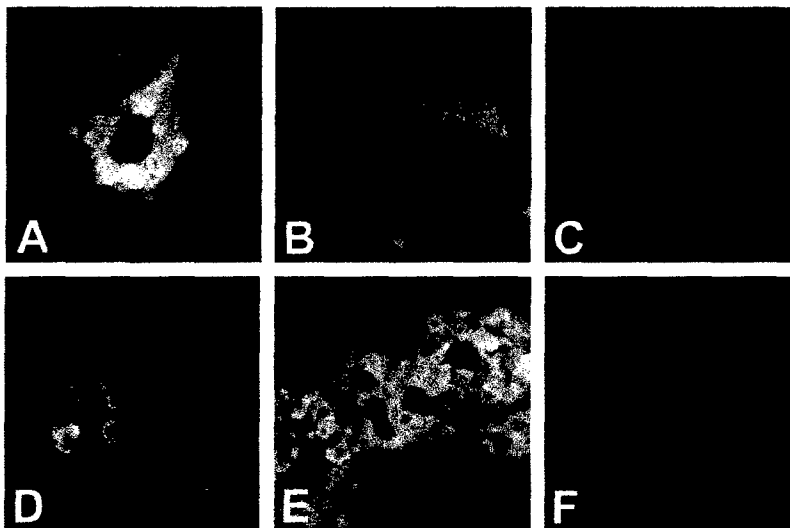


Figure 5

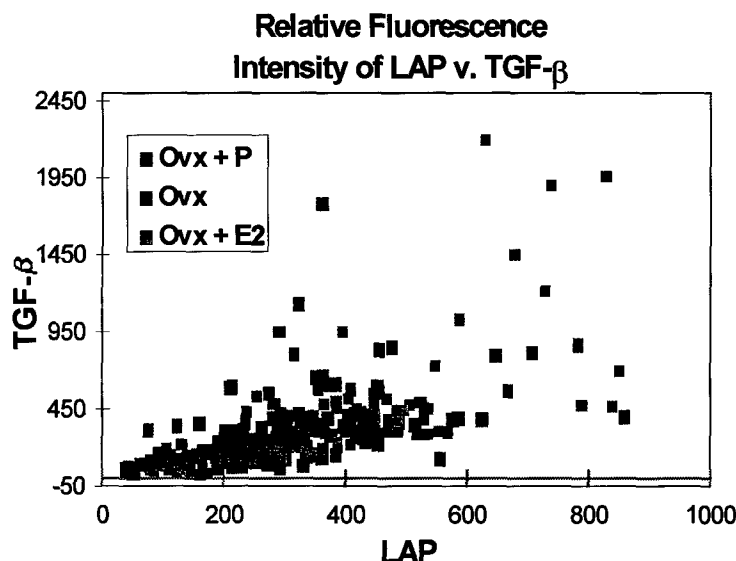


Figure 6

revealed although the intensity of TGF- $\beta$  generally remains low. Thus by using image analysis of dual TGF- $\beta$  and LAP immunostaining we now have a tool to discriminate between production and activation.

LAP immunostaining and contained frequent intensely positive TGF- $\beta$  cells as compared to its control (D). (F: minus antibody control.)

Quantitative analysis of LAP and TGF- $\beta$  immunoreactivity shown in Figure 6 suggest that both production and activation are regulated as a function of hormone treatment. Compared to the ovariectomy control (red), estrogen treatment (green) reduces both TGF- $\beta$  and LAP immunoreactivity. In contrast, progesterone treatment (blue) mainly increases TGF- $\beta$  relative to LAP, which is indicative of activation. These data define at least two populations of TGF- $\beta$ /LAP cells in intact mammary glands: one in which LAP and TGF- $\beta$  seem to be almost linearly related and another in which TGF- $\beta$  intensity is relatively greater than that of LAP. The latter pattern is consistent with activation and the population represents the intensely stained cells. The concomitant increase in both epitopes is consistent with synthesis, during which some epitopes in TGF- $\beta$  are

**Postulated hormonal regulation of TGF- $\beta$  activation and its consequences.** Our hypothesis is that estrogen and progesterone differentially regulate TGF- $\beta$  production and activation in the mammary epithelium as a means of controlling mammary proliferation and guiding morphogenesis. Since estrogen depresses TGF- $\beta$  while progesterone appears to stimulate it, thus far, the following model of estrogen and progesterone action and TGF- $\beta$  production and activity appears consistent: During puberty, the rise of estrogen generally inhibits TGF- $\beta$ , as is evidenced along the ducts. Estrogen induced proliferation is accompanied by acquisition of progesterone receptor (PR), which, in turn, drives increased production and activation of LTGF- $\beta$ . In adults during proestrus, estrogen primes the epithelium for growth by reducing TGF- $\beta$  and stimulating PR, which is now required for lobular-alveolar proliferation. However, when hormone levels drop following estrus, PR and TGF- $\beta$  expression both decrease. If hormone levels remain high, then PR maintains TGF- $\beta$  production and activation. But when proliferation is complete, as occurs at late pregnancy, PR down regulates itself and concomitantly TGF- $\beta$  regulation reverts to estrogen control, which results in its inhibition and decreased frequency of intensely positive TGF- $\beta$  cells.

Based on studies in cell culture models, there are three general consequences of TGF- $\beta$  activation: inhibition of DNA synthesis, induction of apoptosis and modulation of ECM remodeling. In general, we think that low levels of TGF- $\beta$ , present for example during diestrus, are growth inhibitory. Estrogen relieves growth inhibition during puberty and proestrus by reducing TGF- $\beta$  production and activity. PR on the other hand is acquired in endbud cells but fails to respond to progesterone (Bresciani, 1968). We propose that the intense TGF- $\beta$  immunoreactivity found in the endbud is induced by PR and is indicative of its activity as a signal for apoptosis, which is important for morphogenesis of the duct lumen (Humphreys et al., 1996). In endbuds, as is common during morphogenesis of many tissues, proliferation is offset, or edited, by high frequency of apoptosis. In adults however, progesterone actually both augments proliferation and generates a stop signal, i.e. TGF- $\beta$ , that provides balance for lobular-alveolar morphogenesis. If progesterone and estrogen levels remain high then progesterone-induced TGF- $\beta$  may instigate apoptotic remodeling of the lobular-alveolar structure. We found that apoptosis was much higher in early pregnancy than in non-pregnant mammary gland (not shown). A similar increase has been noted in midpregnancy (Kordon et al., 1995). Alternatively, as shown in uterus, PR induced TGF- $\beta$  may be important for ECM remodeling, which is thought to be essential for side-branching.

Since the effects of TGF- $\beta$  depend on the cell type (Westergren-Thorsson et al., 1990), differentiation (Vollberg et al., 1991) and the microenvironment (reviewed in ), the specific consequences of LTGF- $\beta$  activation must be determined within the tissue-specific context to understand and predict the outcome. It is also worth noting that given the heterogeneity of the mammary TGF- $\beta$  immunoreactivity, the action of TGF- $\beta$  may be mediated by concentration. The identity of the cell populations that express estrogen and PR and intense TGF- $\beta$ , indicative of activation, are not known. Whether the distinctive on/off pattern of TGF- $\beta$  intense staining that we observe in endbuds, estrus and early pregnancy reflects autocrine or paracrine regulation by receptor positive cells remains to be determined.

## CONCLUSIONS

Our first year of funding has supported experiments leading to two important and novel observations, while the second year of funding has been spent expanding and confirming these data. We proposed that the effects of carcinogens on the phenotype of host tissue could contribute to the progression

of initiated epithelial cells. We tested whether the abnormal stroma created by radiation exposure promotes the neoplastic potential of COMMA-D mammary cells. These studies provide evidence that supports our hypothesis that the microenvironment elicited by carcinogen exposure can act as a promoter of neoplasia. As a result, the possibility that microenvironments may be a future target for therapeutic intervention or cancer prevention gains credence.

TGF- $\beta$  is a potential mediator of breast cancer progression and an important target for therapy. In order to understand the impact of TGF- $\beta$  action on mammary carcinogenesis it is critical to understand its physiological mechanisms of activation and the consequences of its activity during development. Our initial studies of the physiological regulation of TGF- $\beta$  activation in normal murine mammary gland have shown that activation is highly restricted and is differentially regulated by differentiation and estrus. The postulated role of TGF- $\beta$  as a key regulator of normal mammary proliferation will be examined in using specific hormonal manipulations. Its role in progression will be evaluated using *in vivo* neutralization of TGF- $\beta$  action in radiation chimeric mammary glands. These studies will provide the critical background for determining the consequences of TGF- $\beta$  activation in the irradiated mammary gland and its role in neoplasia.

These experiments have been reported in part in platform presentations at the Era of Hope DOD Breast Cancer meeting (November, 1997), the Keystone Symposia on Breast and Prostate Cancer (February, 1998) and the M.D. Anderson Round Top Conference on Growth Factors (May, 1998). In addition some data were presented in poster format at Radiation Research Society annual meeting (May, 1998). They are presently being prepared for publication.

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